IMMUNOKINASES

The present invention relates to a synthetic, soluble, endogenous complex formed from at least one component A and at least one component B,

5   whereby component A comprises a binding domain for extra-cellular surface structures that internalize upon binding of component A of said complex, and component B has a constitutive catalytic kinase activity and effects cell biosynthesis/signalling including cell death after internalization through phosphorylation. The present invention also relates to nucleic acids and/or

10   vectors coding for such a complex. The present invention furthermore provides a method for influencing the cell growth and/or the physiology of cells to which said complex, nucleic acids or vectors have been targeted. The invention further relates to cells, or cell lines or non-human organisms, such as plants including algae and/or microorganisms, including yeast and fungi,

15   producing the complex of the present invention. The present invention also concerns a kit comprising said complex, nucleic acids, vectors and/or cells. The present invention relates to the use of said complex, nucleic acids, vectors, cells or kit for the manufacturing of a medicament for the treatment of proliferative diseases, allergies, autoimmune diseases and/or chronic

20   inflammation. The present invention further relates to the use of said complex, nucleic acids or vectors, cells and/or kit for targeted modulation of cellular signalling pathways, in order to effect the gene expression, and/or the viability of the target cell in a therapeutic manner. The invention further relates to a medicament comprising said complex, nucleic acids, vectors, cells or

25   organisms. Furthermore the complexes, nucleic acids, vectors, cells and kits of the present invention are usable in prognostic, diagnostic and analytic kinase assays.

Background of the invention

30 · Medications currently available for proliferative diseases, such as chemotherapeutic agents, have the disadvantage of inducing considerable side

effects due to their relative non-specificity. It has been attempted to moderate these by various therapeutic concepts. One potential approach is the use of immunotherapeutic agents to increase the specificity of medication. This approach has been especially useful for the treatment of tumors.

5   One type of an immunotherapeutic agent are immunotoxins. An immunotoxin comprises a monoclonal antibody (moAb) or a recombinant antibody fragment with a specific affinity for surface markers of target cells, which is coupled to a cytotoxic reagent. Cytotoxic agents are selected from toxins or radioactive elements. An immunotherapeutic wherein the cytotoxic agent is a radioactive

10 elements is called radioimmunoconjugate. Immunotoxins and radioimmuno-conjugate have been used for the treatment of malignancies.

Another type of immunotherapeutic agent are anti-immunoconjugates. An anti-immunoconjugate comprises a structure relevant to pathogenesis or a fragment thereof, which is coupled to a toxin component. Anti-

15 immunoconjugates are used for the treatment of autoimmne diseases, tissue reactions or allergies.

When radioactively labeled anti-B-cell moAb were used with B-cell lymphomas, tumor regressions and even complete remissions could be observed (1). In contrast, the results with moAb against solid tumors were rather disillusioning.

20 The relative large size of the ITs used in these studies seemed to interfere with their ability to penetrate the tumors, and made them ineffective therapeutics. The low tumor penetration rate posed a particular challenging problem for poorly vascularized tumors. In order to obtain better tissue and tumor penetration and in general improved diffusion properties, the ITs were

25 miniaturized. It was also speculated, that these smaller ITs would be less immunogenic because of the reduced size of the antigenic determinants (2). Therefore proteolytically cleaved antibody fragments (miniaturized) were conjugated to the above mentioned effector functions (radioactive elements or toxins).

30 Improved cloning techniques allowed the preparation of completely recombinant ITs: Coding regions of immunoglobulin light and heavy chain variable regions, amplified by polymerase chain reaction, are joined together

by a synthetic linker (e.g. (Gly$_4$Ser)$_3$) (SEQ ID NO: 7). The resulting single chain fragment of variable region genes (scFv) is then genetically fused to a coding region of a catalytically active enzyme including cytotoxically active proteins or polypeptides (3).

5   The peptidic cell poisons, which have been mostly used to date and thus best, characterized are the bacterial toxins diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), and the plant-derived Ricin-A (4). The mechanism of cytotoxic activity is essentially the same in all of these toxins despite of their different evolutionary backgrounds. The catalytic domain inhibits protein

10   biosynthesis by direct modification of the elongation factor 2 (EF-2), which is important to translation, or by inactivation of the EF-2 binding site at the 28S-rRNA subunit of ribosomes.

In most of the constructs employed to date, the systemic application of immunotoxins results in more or less severe side effects. In addition to the

15   "vascular leak" syndrome, thrombocytopenia, hemolysis, renal insufficiency and sickness also occur, depending on the construct employed and the applied dosage (4). Dose-dependent liver damage was also observed (5). In addition to the documented side effects, the immunogenicity of the constructs is one of the key problems of immunotherapy. This applies, in particular, to the humoral

20   defense against the catalytic domains employed, such as Ricin (HARA), PE, or DT (2). Theoretically, all non-human structures can provoke an immune response. Thus, the repeated administration of immunotoxins and immunoconjugates is limited. A logical consequence of these problems is the development of human immunotoxins.

25   To date, human toxins used in immunotoxins have in most of all cases been selected from ribonucleases (6). Since human RNases are present in extracellular fluids, plasma and tissues, they are considered less immunogenic when used in immunotoxins. Angiogenin (ANG), a 14 kDa protein having a 64% sequence homology with RNase A, was first isolated from a tumor-cell-

30   conditioned medium, where it was discovered due to its capability of inducing angiogenesis (7). It was shown that the t-RNA-specific RNase activity of Angiogenin has a cytotoxic potential. In accordance with that, chemically

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conjugated immunotoxins subsequently exhibited a cell-specific toxic activity. To evaluate the efficacy of ANG-based immunotoxins, different conformations of ANG with, e.g. epidermal growth factor (EGF) or CD30 ligand, were constructed and successfully tested *in vitro* (8). Another member of the RNase

5   superfamily is eosinophilic neurotoxin (EDN). For EDN, which has a size of 18.4 kDa, only the direct neurotoxicity has been described to date. Based on the documented potency, different EDN-based immunotoxins have been constructed and successfully tested *in vitro* (9).

Very recently it was shown that proteases like granzyme B or derivatives

10   thereof can efficiently fulfill the effector function of immunotoxins (WO – A – 01/80880).

Protein phosphorylation is one of the most important mechanisms by which extracellular signals are transformed into biological responses in cells. Activation of protein kinases is the most common mode of signal transduction

15   in biological systems. The three basic components of the phosphorylation systems are: 1) phosphoproteins that alter their properties by phosphorylation and dephosphorylation; 2) protein kinases that transfer a phosphate group from donor substrates, such as ATP and GTP, to serine, threonine, tyrosine or histidine residues; and 3) protein phosphatases that dephoshorylate

20   phosphorylated proteins, thereby restoring the particular protein phosphorylation system to its basal stage. The eukaryotic protein kinases (ePK) represent the largest superfamily of homologous proteins that are involved in the regulation of intracellular signaling pathways. These kinases phosphorylate amino acid (aa) residues located in the loops or turns of their

25   substrates. To regulate signal transduction pathways, there are approximately 2000 kinases and 500 protein phosphatases encoded within the human genome (10). A large number of these kinases are encoded by oncogenes and tumor-suppressor genes. The primary structures of hundreds of these enzymes are known, and all contain a conserved catalytic core of about 250-

30   300 aa residues. The conserved structural features of the catalytic domain have been found from yeast, lower eukaryotes to mammals. The catalytic domain of a kinase domain is further divided into 12 smaller subdomains,

defined as regions uninterrupted by large insertions and containing characteristic, highly conserved aa residues. Subdomain I-IV, located at the amino-terminus of the catalytic domain, is involved in anchoring and orienting the nucleotide ATP. Subdomains VI-IX form a large lobe structure at the

5 carboxy-terminus of the catalytic domain and are involved in the binding of substrates and catalyzing the phospho-transfer reaction. The pattern of aa residues found within subdomain VIB (HRD motif), VIII (A/SPE motif), and IX (DXWXXG motif (SEQ ID NO. 9) are highly conserved among different protein kinases.

10 The eukaryotic protein kinases make up a large superfamily of homologous proteins (11). A classification scheme is founded on a catalytic domain phylogeny, which reveals families of enzymes that have related substrate specificities and modes of regulation according to the scheme of Hanks and Hunter (12). Most protein kinases contain a conserved catalytic domain

15 belonging to the eukaryotic protein kinase (ePK) superfamily (all other protein kinases are classified as atypical protein kinases (aPKs)). ePK's are classified into seven major groups, and are subdivided into families, and subfamilies, based on the sequence of their ePK domains:

Atypical protein kinases (aPK) lack sequence similarity to the ePK domains,

20 but either have protein kinase activity, or a clear homology of aPKs with protein kinase activity. All aPK families are small, several having just one member in vertebrates. None have been found in invertebrates. A number of reports have shown that the kinases of this subfamily play critical roles in signaling pathways that control cell growth, differentiation and survival.

25 Recently, several investigators have identified a number of aPKC-interacting proteins and their characterization is helping to unravel the mechanisms of action and functions of these kinases. Recently, a new family of aPKs called alpha kinases that does not have any homology to the serine/threonine/tyrosine protein kinase superfamily has been identified (13).

30 The alpha kinases differ from serine/threonine/tyrosine protein kinases in that that they phosphorylate a threonine aa residue located in the alpha helical region of the substrate.

Free calcium is a major second-messenger in all cell types. One mechanism by which calcium ions exert their effects is by binding to a 17-kDa protein, calmodulin (CaM). The binding of four calcium ions to calmodulin changes its conformation and promotes its interaction with a number of other proteins,

5   including several classes of protein kinases that are activated by the calcium/CaM complex (14). Classifying the calcium/CaM-dependent protein kinases is based on their substrate specificity. Some of these enzymes have only one substrate, and are designed as "*dedicated*" calcium/CaM-dependent protein kinases, while others have broad substrate specificity and are termed

10   "*multifunctional*" kinases. The *dedicated* calcium/CaM-dependent protein kinases comprise three enzymes. Phosphorylase kinase, myosin light chain kinase and eukaryotic elongation factor-2 kinase. Multifunctional calcium/CaM-dependent protein kinases comprise four enzymes referred to as CaM-kinases I, II, IV and pro-apoptotic serine/threonine death protein kinases.

15   One of the positive mediators of apoptosis is DAP-kinase (DAPk) (15). DAPk is a pro-apoptotic calcium/CaM-regulated serine/threonine kinase with tumor-suppressive activity. DAPk is frequently inactivated by promoter methylation in human cancer. Its expression is frequently lost in human carcinoma and B-and (NK)/T-cell malignancies, in some cases in association with more

20   aggressive stages of disease (16). Very recently, it has been shown, that no expression of DAPk was detectable in high-metastatic lung carcinoma cell lines, whereas the low-metastatic counterparts were positive for DAPk. Four additional kinases that have a significant homology in their catalytic domain to DAPk were recently identified. ZIP(Dlk)-kinase and DRP-1, also named DAPk2,

25   are the closest family members, as their catalytic domains share approximately 80% identity to that of DAPk. Two more distant DAPk-related proteins are DRAK1 and DRAK2. Both the pro-apoptotic and tumor-suppressive functions of DAPk depend on its kinase catalytic activity. The CaM-regulatory segment of DAPk possesses an autoinhibitory effect on the catalytic

30   activity, and is relieved by binding to Ca2+ -activated CaM. Consistently, the deletion of this segment from DAPk-ΔCaM mutant generated a constitutively active kinase ("super-killing kinase"), which displayed CaM-independent

substrate phosphorylation *in vitro* and promoted apoptotic activity in *vivo* (17). Eukaryotic elongation factor-2 kinase (eEF-2k) belongs to the alpha kinases and is distinct from the main family of protein kinases with which they share no sequence similarity (18). The activity of eukaryotic elongation factor-

5 2 (eEF-2) is crucial for the elongation step of mRNA translation. eEF-2 activity is regulated by phosphorylation. To be active, eEF-2 must be dephosphorylated, since phosphorylation at Thr-56 and 58 causes inactivation, resulting in the termination of mRNA translation. Phosphorylation of eEF-2 at Thr-56 and 58 by the highly specific calcium/CaM-dependent eEF-2k results in

10 eEF-2 inactivation and, therefore, may regulate the global rate of protein synthesis at the elongation stage in animal cells. eEF-2k is itself regulated both negatively and positively by phosphorylation on at least five different serine residues, probably mediated by seven or more protein kinases. Very recently, it has been shown, that a point mutation at Ser-499, eEF-2K S499D,

15 transforms the kinase into a constitutively active form (19).

Protein phosphorylation is implicated in cellular processes such as proliferation, differentiation, secretion, invasion, angiogenesis, metastasis and apoptosis. Protein kinases and phosphatases play key roles in regulating these processes. Changes in the level, subcellular location and activity of kinases

20 and phosphatases have consequences on normal cell function and maintenance of cellular homeostasis. Dysfunction in activities of protein kinases may lead to severe pathological states. In cancer, as well as in other proliferative diseases, deregulated cell proliferation, differentiation and survival frequently results from abnormal protein phosphorylation.

25 The identification of the key roles of protein kinases in proliferative diseases has led to extensive efforts to develop kinase inhibitors for treatment of a wide range of cancers. Many different tyrosine and serine/threonine protein kinases have been selected as candidates for drug discovery activities in oncology/inflammatory research, based either on their overexpression and/or

30 on dysfunction in a particular organ or tissue, or through their association in deregulated signal transduction/cell cycle pathways. To date, more than 30 different tyrosine kinase targets are under evaluation in drug discovery

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projects in oncology. Chemical inhibitors (organic molecules, peptide inhibitors), antisense oligonucleotides and kinase-selective antibodies have been developed which target intracellular kinases.

Nevertheless, development was slow and associated with problems, mainly
5   because of the associated toxicity, attributed to the poor selectivity of these compounds. Protein kinase inhibitors mainly bind at the active site of the enzyme, in competition with ATP+, and whether such inhibitors could ever be used for the long-term treatment of chronic conditions, such as rheumatoid arthritis, is still questionable.

10   Similarly the state of the art immunotoxins, such as chemically-linked or recombinant immunotoxins comprising ribonucleases, are still associated with the problem of unspecific toxicity. This problem reduces the efficiency of compositions comprising said immunotoxins, and limits their usefulness as therapeutic agents.

15   Very recently, different chimeric proteins of kinases fused to distinct ligands were developed: A) Ligand-kinase fusion proteins were constructed to influence T-cell behaviour after transfection (US-A-5,670,324): after transformation of T-cells with a vector coding for a chimeric CD4-kinase fusion, the expressed chimeric membrane-bound molecules may be used to
20   identify drugs that block T cell activation or low level self-antigens. B) Chimeric kinase-based receptors were also constructed to redirect immune effector cells. Human immune effector cells transformed with a vector encoding for a membrane-bound ligand-kinase fusion proteins may be able to specifically target cells via their extracellular ligand and may initiate killing of
25   the target cells by activity of the fused kinase acticity triggering activation of the transformed immune effector cell (US 2002/0176851 A1). C) Cyclin dependent kinases (CDKs), in particular human Myt-1 kinase and derivatives therof were fused to the constant region of immunoglobulin molecules, which may improve pharmokinetic properties and simplify expression and purification
30   of Myt-1 (US 5,935,835). D) Other kinase-based fusion proteins, in particular scFv-kinase fusion proteins were constructed for the indirect identification of

protein-protein interactions inside living cells after their transformation with two different vectors (US 2002/0151684 A1).

None of these kinase fusions is available as a soluble protein that would allow their use as a human immunotoxin.

5 Surprisingly it was found that the above-mentioned problems can be solved by soluble, endogenous complexes comprising cell-specific antibody fragment(s) which is/are linked to constantly and catalytically active kinase(s) that develop cytotoxic/regulative activity upon internalization of the complex. Surprisingly, the complexes of the present invention are superior over state of the art

10 immunotoxins in that they have a higher specificity combining specific binding to a target cell with specific constitutive catalytic activity inside the target cell, a reduced immunogenicity, an improved activity and are resistant to non-specific inactivation, and are thus are less prone to activity reduction.

15 Summary of the invention

The present invention concerns a synthetic complex formed from at least one component A and at least one component B, whereby component A comprises a binding domain for extra-cellular surface structures that internalize upon binding of component A of said complex , and component B has constitutivly a

20 catalytic kinase activity, said complex is soluble and effects cell death after internalization. The component A is selected from the group of actively binding structures consisting of antibodies or their derivatives or fragments thereof, and/or chemical molecules such as carbohydrates, lipids, nucleic acids, peptides, vitamins, etc., and/or small molecules with up to 100 atoms with

25 receptor-binding activity such as ligands, in particular single ions, peptidic molecules, non-peptidic molecules, etc., and/or cell surface carbohydrate binding proteins and their ligands such as lectins, in particular calnexins, c type lectins, l-type lectins, m-type lectins, p-type lectins, r-type lectins, galectins and their derivatives, and/or receptor binding molecules such as

30 natural ligands to the cluster of differentiation (CD) antigens, like CD30, CD40, etc., cytokines such as chemokines, colony stimulating factors, type-1

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cytokines, type-2 cytokines, interferons, interleukins, lymphokines, monokines, etc., and/or adhesion molecules including their derivatives and mutants, and/or derivatives or combinations of any of the above listed of actively binding structures, which bind to CD antigens, cytokine receptors, hormone receptors, growth factor receptors, ion pumps, channel-forming proteins. The component A may also be selected from the group of passively binding structures consisting of allergens, peptidic allergens, recombinant allergens, allergen-idiotypical antibodies, autoimmune-provoking structures, tissue-rejection-inducing structures, immunoglobulin constant regions and their derivatives, mutants or combinations thereof. The complex of the present invention is directed by its component A to a target cell comprising a binding partner for the above listed binding structures of A. In a further embodiment the component A of the complex has a higher valency by comprising two or more identical and/or different binding structures. The complex of the present invention also comprises a component B which is at least one kinase selected from the following three classes of kinases: 1. eukaryotic protein kinase (ePK) superfamily, 2. histidine protein kinase (HPK) superfamily or 3. atypical protein kinase (aPK) superfamily. In a further embodiment the component B is a human kinase or a non-human kinase. A further embodiment of the invention is a complex wherein the ePK is selected from the group of calcium/calmodulin-regulated (CaM) death-promoting kinases, consisting of death-associated protein kinase (DAP-kinase, DAPk), DAP kinase-related protein kinase 1 (DRP-1), also named DAP-kinase 2 (DAPk2), DAP like kinase/Zipper Interacting protein kinase (Dlk/ZIP-kinase), also named DAP-kinase 3 (DAPK3) and DAP kinase related apoptosis-inducing kinase (DRAK1 and DRAK2) families, the group of Group of calcium/calmodulin-regulated (CaM) death-promoting kinases-like (CAMKL) family, consisting of at least 49 subfamilies, protein kinase AMP-activated alpha 1 catalytic subunit (PRKAA1), protein kinase AMP-activated alpha 2 catalytic subunit (PRKAA2), BRSK1 and BRSK2, CHK1 checkpoint homologue (CHEK1), hormonally upregulated Neu-associated kinase (HUNK), serine/threonine kinase 11 (Peutz-Jeghers syndrome) (STK11), MAP/microtubule affinity-regulating kinase (MARK) 1-4,

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MARKps 01-30, likely ortholog of maternal embryonic leucine zipper kinase (KIAA0175), PAS domain containing serine/threonine kinase (PASK), NIM1, QIK and SNRK, the group of death-domain receptor interacting protein kinase (RIP-kinase) family, consisting of at least six subfamilies, RIP-kinase 1, RIP-
5   kinase 2, RIP-kinase 3 and RIP-kinase 4, ankyrin repeat domain 3 (ANKRD3) and SqK288, the group of multifunctional CaM kinase family, consisting of CaM kinases I, II, including the microtubule affinity-regulating kinases (MARK) and microtubule affinity-regulating kinases-like 1 (MARKL1), CaM kinase IV and CaM kinase kinase subfamilies, the group of dedicated CaM kinases, consisting
10   of Myosin light chain kinase (MLCk), phosphorylase kinase and CaM kinase III (eEF-2k), the group of mitogen-activated protein kinase (MAPK) family, consisting of extracellular signal-regulated kinases (ERK), c-JUN NH2-terminal protein kinases (JNK), nemo-like kinase (NLK) and p38 kinase subfamilies, the group of cyclin-dependent kinase (CDK) family, consisting of the subfamilies,
15   cell cycle related kinase (CCRK), cell division cycle 2 (CDC2), cyclin-dependent kinases (CDK) 1-11, PCTAIRE protein kinase (PCTK) 1-3, PFTAIRE protein kinase (PFTK) 1-2 and cell division cycle 2-like 1 (PITSLRE proteins), the group of eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3) family, also named (PEK), consisting of the protein kinase interferon-inducible double
20   stranded RNA (dsRNA) dependent (PKR) subfamily. A further embodiment of the present invention concerns a complex wherein the histidine protein kinase is selected from one of the eleven families HPK 1-11. A further embodiment of the present invention is a complex wherein the aPK is selected from the alpha protein kinase family, consisting of eukaryotic elongation factor-2 kinase (eEF-
25   2k), myosin heavy chain kinase (MHC-kinase), eukaryotic translation initiation factor 2 alpha kinase 1 (E2K1) and channel kinase (Chak1 and Chak2) subfamilies, the group of Fas-activated s/t kinase (FASTK) family, consisting of the FASTK subfamily, the group of protein tyrosine kinase 9 (A6) family, consisting of A6 and protein tyrosine kinase 9-like (A6r) subfamilies, the group
30   of p21-activated protein kinases (PAK) family, consisting of the three highly conserved isoforms: alpha-PAK (PAK1), beta-PAK (PAK3) and gamma-PAK (PAK2, PAKI), the group of Interleukin-1 (IL-1)-receptor-associated kinase

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(IRAK) family, consisting of IRAK-1, IRAK-2, IRAK-3 and IRAK-4 subfamilies, or derivatives, mutants or combinations thereof. These kinases were selected because they maintain their activity in a soluble complex. A further embodiment is a complex wherein the kinase activity of component B directly

5 activates or inactivates components of cell-regulatory pathways through phosphorylation, acetylation, methylation, prenylation, and sulfation, altering the function, gene expression, or viability of a target cell, whereby a target cell is defined by the ability of component A to bind to the cell. Preferably the component B activates or inactivates components of cell-regulatory pathways

10 through phosphorylation. In a further embodiment, component B of the complex is DAPK2 or a derivative thereof or EF-2K or a derivative thereof. Those two kinases were found to be particular effective in a complex according to the present invention. A further advantage of the DAPK2 is the existence of a constitutive active mutant of said enzyme which is particular suitable for the

15 complex of the present invention. DAPKs are frequently found to be inactivated in human tumor cells. The complex of the present invention comprising such a DAPK is therefore particular useful since it enables the reintroduction of an active DAPK into, for example, a tumor. A complex comprising eEF-2k as component B has the advantage that it will be active in

20 any human cell, since eEF-2k is ubiquitous. A derivative of those kinases is defined as a constitutively active kinase which has accumulated at least one mutation and/or modification, i.e. a deletion, a substitution, a domain swapping, etc. Preferred mutations are conservative amino acid changes, and preferred modifications are phosphorylations, acetylations, methylations etc.

25 A further embodiment of the present invention is a complex comprising one or more supplementary components S which regulate protein biosynthesis on the transcription and/or translation level, and/or enable purification and/or detection of the complex or its components, and/or facilitate translocation of at least component B into the target cell and intracellular separation therein,

30 and/or activation of component B. A further embodiment of the present invention is a complex wherein the components are chemically coupled and/or genetically fused to each other. A further embodiment are the genetically

fused complexes named *L-DAPk2-Ki-4-III/G* (SEQ ID NO: 2), *Ki-4-DAPk2-II/G* (SEQ ID NO: 4) and Ki-4(scFv)-eEF-2K (SEQ ID NO: 6), encoded by the corresponding DNA molecules with SEQ ID NOs 1, 3, and 5, respectively. A further embodiment of the present invention are a nucleic acid molecule

5   coding for said complex or for individual components thererof for the preparation of such complex, and/or a vector comprising said nucleic acid molecule. The present invention also concerns cells and non-human organisms synthesizing complete complexes or individual components thereof after having been transformed or transfected with nucleic acid molecules coding for

10 said complexes of the present invention, or *in vitro* translation systems synthesizing complete complexes or individual components thereof. A further embodiment are also an organism and/or a cell transformed or transfected with the nucleic acid molecule or vector encoding said complex or components thereof, whereby said organism is either a prokaryote, such as *E. coli, B.*

15 *subtilis, S. carnosus, S. coelicolor,* and/or *Marinococcus sp.,* or a lower eukaryote, such as *Saccharomyces sp., Aspergillus sp., Spodoptera sp.* and/or *P. pastoris,* or a higher non-human eukaryote such as a plant and/or an animal, and the cell is a primary or cultivated mammalian cell, such as a freshly isolated human cell or a eukaryotic cell line, such as CHO, Cos or 293.

20 A further embodiment is a method for influencing the growth and/or the physiology of the cells transfected or transformed with the nucleic acid molecule or the vector encoding said complex, by culturing the cells under conditions supporting the activity of the complex. A further embodiment of the present invention is a kit comprising the complex and/or the nucleic acid

25 molecule and/or the vector, and/or the cells and/or prokaryotes and/or lower eukaryotes transfected or transformed with said nucleic acid molecules of the present invention. A further embodiment is the use of the complex, and/or the nucleic acid molecules, and/or vectors, and/or the cells and/or prokaryotes and/or lower eukaryotes transfected or transformed with said nucleic acid

30 molecules and/or the kit for the preparation of a medicament for the treatment of proliferative diseases, such as cancerous or non-cancerous

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proliferative diseases, allergies, autoimmune diseases and/or chronic inflammation.

A further embodiment is a medicament comprising a complex, and/or nucleic acid molecules and/or vectors and/or or cells or organisms synthesising the

5   complex of present invention, for treating proliferative diseases, such as cancerous or non-cancerous proliferative diseases, allergies, autoimmune reactions, chronic inflammation reactions or tissue rejection reactions. A further embodiment is the *ex vivo, in vivo or in vitro* use of the complex, and/or the nucleic acid molecule and/or the vector, and/or the cells and/or the

10   organisms synthesising the complex and/or the kit, for the targeted modulation of cellular signaling pathways. A further embodiment is the use of the complex, and/or the nucleic acid molecule and/or the vector, and/or the cells and/or organisms synthesising the complex and/or the kit for prognostic, diagnostic, and/or analytic kinase assays, and/or for the the development of

15   such assays. A further embodiment is a method of treatment of proliferative diseases, such as cancerous or non-cancerous proliferative diseases, allergies, autoimmune diseases, and/or chronic inflammation comprising the steps of administering to a patient the complex of the present invention and/or the nucleic acid and/or the vector encoding said complex.

20

Brief description of the drawings

Figure 1: Cloning of pMS-(L-DAPk2-Ki-4)-III/G (SEQ ID NO 1), pMS-(Ki-4-DAPk2)-II/G (SEQ ID NO 3) and pMT-Ki-4(scFv)-eEF-2K (SEQ ID NO 5). Lane 1-3, PCR-amplification of DAPk2 and derivatives thereof. Lane 4, PCR-

25   amplification of eEF-2K and derivatives thereof. (M, DNA-ladder; C, negative control).

Figure 2: Schematic structure of the eukaryotic expression cassettes pMS-(L-DAPKk2-Ki-4)-III/G (SEQ ID NO 1), pMS-(Ki-4-DAPk2)-II/G (SEQ ID NO 3) and prokaryotic expression module pMT-Ki-4(scFv)-eEF-2K coding region.

30   Legends: hCMV = human Cyto-megalovirus promotor/enhan-cer; Ig-*k*-L = Immunoglobin kappa-chain leader sequence; M / H = c-Myc epitope

(EQKLISEEDL (SEQ ID NO: 8)) and *hexa*-Histidine tag; IVS / IRES = Intervening sequence / internal ribosome entry site; EGFP = enhanced green fluorescent protein; T7-lac = bacteriophage T7 promotor-lactose operator; pelB = bacterial leader/signal sequence pectate lyase B from *Erwinia*

5   *carotovora* EC; His_{10} = *deca*-Histidine tag; V_H = Immunoglobulin variable heavy-chain; V_L = Immunoglobulin variable light-chain; $(G_4S)_3$ = (Glycine x 4 – serine) x 3 linker; ATG = Translation initiation codon; Stop = Translation termination codon; DAPK2 = Death-associated protein-kinase 2 / DRP-1; eEF-2K = eukaryotic elongation factor-2 kinase; Ki-4 = anti-CD30 immunoglobulin

10   single-chain variable fragment (scFv).

Figure 3: Binding properties of the recombinant anti-CD30 immunokinases. Binding of pMS-(L-DAPk2-Ki-4)-III/G (SEQ ID NO 2) to CD30-positive cells by flow cytometry. Cells were stained with purified Immunokinase (B) or with PBS as negative control (A).

15   Figure 4: Growth inhibition of Hodgkin-derived CD30-positive cell lines after incubation with pMS-(L-DAPk2-Ki-4)-III/G as documented by cell-viability assays. L-540Cy cells were treated with different dilutions of recombinant ani-CD30 immunkinase, and their ability to metabolize the XTT to a water-soluble formazan salt was measured as absorbance at 450 and 650 nm.

20   Measurements were performed in triplicate. Results are presented as percentage of untreated control cells and to Zeocin-treated positive control.

Figure 5: Nucleic Acid sequence of the construct pMS-(L-DAPK2'-Ki-4)-III/G open reading frame (ORF).

Figure 6: Amino acid sequence of the construct pMS-(L-DAPK2'-Ki-4)-III/G

25   open reading frame (ORF).

Figure 7: Nucleic acid sequence of the construct pMS-(L-DAPK2'-Ki-4)-II/G ORF.

Figure 8: Amino acid sequence of the construct pMS-(L-DAPK2'-Ki-4)-II/G ORF.

30   Figure 9: Nucleic acid sequence of the construct pMT-Ki4 (scFv)-eEF-2K ORF.

Figur 10: Amino acid sequence of the construct pMT-Ki4 (scFv)-eEF-2K ORF.

Figur 11: Amino acid sequence of the synthetic linker.

Figure 12: Amino acid sequence of the c-Myc epitope.

Figure 13: Motif in the domain IX of kinases.

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Detailed description of the invention

The complex according to the invention is a recombinant heterologous complex comprising at least two domains, i.e. one effector domain and at least one cell-specific binding domain. The complex according to the invention is
10   usable for diagnosis and therapy of diseases.

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications and applications, cited previously or below are hereby
15   incorporated by reference.

Definitions

As used herein, the term "immunotoxin" refers to chimeric molecules in which a cell-binding monoclonal antibody or fragments thereof are chemically
20   coupled or genetically fused to toxins or their subunits. The toxin portion of the immunotoxin can be derived form various sources, such as plants, animals, higher and lower microorganisms such as bacteria and fungi, and in particular if the toxin is a catalytic enzyme, the enzyme can be of human origin. The toxin can also be a synthetic drug. Immunotoxins as well their
25   constructions are reviewed above and are well known to the person skilled in the art.

As used herein, the term "immunokinase" refers to chimeric molecules in which a cell-binding monoclonal antibody or fragments thereof are coupled or fused to kinases or their subunits harboring the kinase activity. The term
30   immunokinase is a synonym for the complex of the present invention.

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As used herein, the term "component A" of the complex represents the actively binding structure of the complex of present invention. The component A is selected from the group of actively binding structures consisting of antibodies or their derivatives or fragments thereof, synthetic peptides such as

5   scFv, mimotopes, etc. or chemical molecules such as carbohydrates, lipids, nucleic acids, peptides, vitamins, etc., and/or small molecules with up to 100 atoms with receptor-binding activity like ligands, in particular single atoms, peptidic molecules, non-peptidic molecules, etc., and/or cell surface carbohydrate binding proteins and their ligands such as lectins, in particular

10   calnexins, c-type lectins, I-type lectins, m-type lectins, p-type lectins, r-type lectins, galectins and their derivatives, and/or receptor binding molecules such as natural ligands to the cluster of differentiation (CD) antigens, like CD30, CD40, etc., cytokines such as chemokines, colony stimulating factors, type-1 cytokines, type-2 cytokines, interferons, interleukins, lymphokines,

15   monokines, etc., and/or adhesion molecules including their derivatives and mutants, and/or derivatives or combinations of any of the above listed of actively binding structures, which bind to CD antigens, cytokine receptors, hormone receptors, growth factor receptors, ion pumps, channel-forming proteins. The component A may also be selected from the group of passively

20   binding structures consisting of allergens, peptidic allergens, recombinant allergens, allergen-idiotypical antibodies, autoimmune-provoking structures, tissue-rejection-inducing structures, immunoglobulin constant regions and their derivatives, mutants or combinations thereof. A component A with higher valency may be generated by combining at least two identical or different

25   binding structures selected from the above mentioned groups.
As used herein, the term "antibody" refers to polyclonal antibodies, monoclonal antibodies, humanized antibodies, single-chain antibodies, and fragments thereof such as Fab, F(ab')2, Fv, and other fragments which retain the antigen binding function and specificity of the parent antibody.

30   As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be

limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')2, Fv, and others which retain the antigen binding function and specificity of the antibody. Monoclonal antibodies of any mammalian species can be used in this

5   invention. In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

As used herein, the term "human antibodies" means that the framework

10 regions of an immunoglobulin are derived from human immunoglobulin sequences.

As used herein, the term "single chain antibody fragments" (scFv) refers to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety, which permits

15 preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding to the antigen. Determination and construction of single chain antibodies are described in U.S. Pat. No. 4,946,778 to Ladner et al.

The "component B" of present invention represents the "targeted kinase"

20 moiety of the immunokinase of the present invention and may be selected from any kinase known in the art. Presently, over 5,000 kinase-like sequences from diverse species are available for analysis in public databases. The human genome appears to encode 510 protein kinases in addition to many pseudo-protein kinase genes, and these have been subclassified into over 57 families.

25 There may well be additional protein kinases that remain to be identified (http://www.kinexus.ca/kinases.htm). However, preferably component B is chosen from the following three classes of kinases, which are all known to be active in humans and to retain their kinase activity in a soluble complex. 1. The eukaryotic protein kinase (ePK) superfamily, 2. the histidine protein

30 kinase (HPK) superfamily, or 3. the atypical protein kinase (aPK) superfamily. If component B is chosen from the ePK superfamily, it is selected from the group of calcium/calmodulin-regulated (CaM) death-promoting kinases,

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consisting of death-associated protein kinase (DAP-kinase, DAPk), DAP kinase-related protein kinase 1 (DRP-1), also named DAP-kinase 2 (DAPk2), DAP like kinase/Zipper interacting protein kinase (Dlk/ZIP-kinase), also named DAP-kinase 3 (DAPK3) and DAP kinase related apoptosis-inducing kinase (DRAK1 and DRAK2) families, the group of calcium/calmodulin-regulated (CaM) death-promoting kinases-like (CAMKL) family, consisting of at least 49 subfamilies, protein kinase AMP-activated alpha 1 catalytic subunit (PRKAA1), protein kinase AMP-activated alpha 2 catalytic subunit (PRKAA2), BRSK1 and BRSK2, CHK1 checkpoint homologue (CHEK1), hormonally upregulated Neu-associated kinase (HUNK), serine/threonine kinase 11 (Peutz-Jeghers syndrome) (STK11), MAP/microtubule affinity-regulating kinase (MARK) 1-4, MARKps 01-30, likely ortholog of maternal embryonic leucine zipper kinase (KIAA0175), PAS domain containing serine/threonine kinase (PASK), NIM1, QIK and SNRK, the group of death-domain receptor interacting protein kinase (RIP-kinase) family, consisting of at least six subfamilies, RIP-kinase 1, RIP-kinase 2, RIP-kinase 3 and RIP-kinase 4, ankyrin repeat domain 3 (ANKRD3) and SqK288, the group of multifunctional CaM kinase family, consisting of CaM kinases I, II, including the microtubule affinity-regulating kinases (MARK) and microtubule affinity-regulating kinases-like 1 (MARKL1), CaM kinase IV and CaM kinase kinase subfamilies, the group of dedicated CaM kinases, consisting of Myosin light chain kinase (MLCk), phosphorylase kinase and CaM kinase III (eEF-2k), the group of mitogen-activated protein kinase (MAPK) family, consisting of extracellular signal-regulated kinases (ERK), c-JUN NH2-terminal protein kinases (JNK), nemo-like kinase (NLK) and p38 kinase subfamilies, the group of cyclin-dependent kinase (CDK) family, consisting of the subfamilies, cell cycle related kinase (CCRK), cell division cycle 2 (CDC2), cyclin-dependent kinases (CDK) 1-11, PCTAIRE protein kinase (PCTK) 1-3, PFTAIRE protein kinase (PFTK) 1-2 and cell division cycle 2-like 1 (PITSLRE proteins), the group of eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3) family, also named (PEK), consisting of the protein kinase interferon-inducible double stranded RNA (dsRNA) dependent (PKR) subfamily.

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If component B is chosen from the HPK superfamily, it is selected from the group of at least eleven families HPK 1-11.

If component B is chosen from the aPK superfamily, it is selected from the group of alpha protein kinase family, consisting of eukaryotic elongation
5   factor-2 kinase (eEF-2k), myosin heavy chain kinase (MHC-kinase), eukaryotic translation initiation factor 2 alpha kinase 1 (E2K1) and channel kinase (Chak1 and Chak2) subfamilies, the group of Fas-activated s/t kinase (FASTK) family, consisting of the FASTK subfamily, the group of protein tyrosine kinase 9 (A6) family, consisting of A6 and protein tyrosine kinase 9-like (A6r) subfamilies,
10   the group of p21-activated protein kinases (PAK) family, consisting of the three highly conserved isoforms: alpha-PAK (PAK1), beta-PAK (PAK3) and gamma-PAK (PAK2, PAKI), the group of Interleukin-1 (IL-1)-receptor-associated kinase (IRAK) family, consisting of IRAK-1, IRAK-2, IRAK-3 and IRAK-4 subfamilies.

15   The term "target cell" and or "target tissue" refers to cells or tissues carrying an extracellular surface structure to which the component A of the complex actively or passively binds. Target cells and target tissues are thus cells and tissues to which the component A of the complex can bind. The target cells and target tissues are further characterized by their ability to internalize the
20   complex according to the present invention upon binding of component A.

The term "soluble" refers to the ability of the complex to stay in solution when recombinantly expressed, in particular during protein purification, enabling high yields. The term "soluble" also refers to the state of the complex in fluidic systems inside an organism, until specifically attached to the target cell/tissue.
25   The term also refers to the state of the complex inside a cell upon release from any kind of incorporation vesicles.

The term "endogenous" refers to the localization of the complex in the surrounding/environment of a given target cell/tissue.

The term synthetic refers to a man-made complex, not found in nature. The
30   term also comprises the meaning of "recombinant".

The term "recombinant" refers to the preparation of molecules, in particular the covalent joining of molecules from different sources, by any one of the

known methods of molecular biology. As used in the present invention, the term "recombinant" refers in particular to the fusion of the antibody part to the toxin part by any one of the known methods of molecular biology, such as through production of single chain antibodies. The recombinant DNA molecule encoding the recombinant fusion protein comprising the antibody part and the toxin part are recombinantly expressed. Recombinant immunotoxin produced in this way may be isolated by any technique known in the field of recombinant DNA expression technology suitable for this purpose.

The term "derivative" refers to a mutated or modified protein which has retained its characterizing activity, i.e. binding activity or kinase activity. Particular preferred are constitutively active derivatives. The term derivative comprises proteins which carry at least one amino acid substitution, deletion, addition, a swapping of a single domain or at least one modification of at least one amino acid. Preferred are derivatives which carry 20 such changes, more preferred are those with 10 such changes and most preferred are those with 1 to 5 such changes. Modifications, which can occur, are phosphorylation, acetylation, methylation, prenylation and sulfation.

As used herein, the term "vector" comprises DNA and RNA forms of a plasmid, a cosmid, a phage, phagemid, derivatives of them, or a virus. A vector comprises control sequences and coding sequences.

The term "expression of the recombinant genes encoding the recombinant complex", wherein the recombinant complex is a single chain antibody-toxin moiety fusion polypeptide, also called recombinant immunokinase, refers to the transformation and/or transfection of a host cell with a nucleic acid or vector encoding such a complex, and culturing said host cells selected from the group of bacteria, such as *E. coli, and/or* in yeast, such as in *S. cerevisiae,* and/or in established mammalian or insect cell lines, such as CHO, COS, BHK, 293T and MDCK cells, and/or in primary cells, such as human cells, non-human vertebrate cells, and/or in invertebrate cells such as insect cells, and the synthesis and translation of the corresponding mRNA, finally giving rise to the recombinant protein, the recombinant complex. In more detail, the term

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"expression of the recombinant genes encoding the recombinant complex", comprises the following steps:

Transformation of an appropriate cellular host with a recombinant vector, in which a nucleotide sequence coding for the fusion protein had been inserted

5   under the control of the appropriate regulatory elements, particularly a promoter recognized by the polymerases of the cellular host. In the case of a prokaryotic host, an appropriate ribosome binding site (RBS) also precedes the nucleotide sequence coding for the fusion protein, enabling the translation in said cellular host. In the case of an eukaryotic host any artificial signal

10 sequence or pre/pro sequence may be provided, or the natural signal sequence may be employed. The transformed cellular host is cultured under conditions enabling the expression of said insert.

As used herein, the expression "killing of antigen-expressing cells" refers to the inhibition of protein synthesis or induction of apoptosis, resulting in

15 elimination or death of these cells.

The term "supplementary components S", refers to an additional component of the complex comprising A and B. The supplementary component S contributes features and properties to the complex which allow efficient preparation and/or modify the effectiveness of the complex:

20 – the inducible regulation of transcription/translation (e.g., inducible promoters);

– control of protein biosynthesis (e.g., leader sequences);

– purification/detection of the complex or its components (e.g., His tag, affinity tags);

25 – translocation of the apoptotic agents into the target cells (e.g., translocation domain, amphiphatic sequences);

– intracellular activation/separation of component B (synthetic pro-granzyme B, amphiphatic sequences).

30 Thus the component S is selected from the group of inducible promoters, leader sequences, affinity tags, His tags, translocation domain, amphiphatic sequences and synthetic pro-granzyme B. The invention also relates to nucleic

acid molecules, such as DNA and/or RNA, or vectors, which code for the complex of the present invention or for individual components for preparing the complex. The feasability of the expression of the nucleic acids encoding a recombinant complex in eukaryotic cells of human origin is successfully

5   documented here, as well as the feasibility to use the complex as an specific apoptotic agents in eukaryotic cells of human origin. This suggests the suitability of nucleic acids coding for a complex according to the invention also for non germ line gene-therapeutic approaches. A person skilled in the art is capable of recognizing the various aspects and possibilities of gene-therapeutic

10   interventions in connection with the various diseases to be treated. In addition to the local application of relatively non-specific vectors (e.g., cationic lipids, non-viral, adenoviral and retroviral vectors), a systemic application with modified target-cell-specific vectors will also become possible in the near future. Complexes and nucleic acid molecules and/or vectors coding for the

15   complexes of present invention, are used for the preparation of medicaments for non-germ line gene therapeutic interventions, for the local or systemic application. An interesting alternative to systemic application are the well-aimed *ex vivo* transfection of defined cell populations and their return into the organism, or the use of the *ex vivo* transfected defined cell populations for the

20   preparation of a medicament for the treatment of diseases associated with these cell populations.

Also claimed are cells or *in vitro* translation systems, which synthesize complete complexes according to the invention or individual components thereof, after transformation and/or transfection with, or addition of the

25   nucleic acid molecules or vectors according to the invention.

Cells or organisms according to the invention are either of prokaryotic origin, especially from *E. coli*, *B. subtilis*, *S. carnosus*, *S. coelicolor*, *Marinococcus sp.*, or eukaryotic origin, especially from *Saccharomyces sp.*, *Aspergillus sp.*, *Spodoptera sp.*, *P. pastoris*, primary or cultivated mammalian cells, eukaryotic

30   cell lines (e.g., CHO, Cos or 293) or plants (e.g. *N. tabacum*).

The invention also relates to medicaments comprising the complex according to the present invention and/or the nucleic acid or vectors encoding the

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complex of present invention. Typically, the complexes according to the invention are administered in physiologically acceptable dosage forms. These include, for example, Tris, NaCl, phosphate buffers and all approved buffer systems, especially including buffer systems, which are characterized by the

5   addition of approved protein stabilizers. The administration is effected, in particular, by parenteral, intravenous, subcutaneous, intramuscular, intratumoral, transnasal administrations, and by transmucosal application.

The dosage of the complexes according to the invention to be administered must be established for each application in each disease to be newly treated

10   by clinical phase I studies (dose-escalation studies).

Nucleic acids or vectors, which code for a complex according to the invention, are advantageously administered in physiologically acceptable dosage forms. These include, for example, Tris, NaCl, phosphate buffers and all approved buffer systems, especially including buffer systems, which are characterized by

15   the addition of approved stabilizers for the nucleic acids and/or vectors to be used. The administration is effected, in particular, by parenteral, intravenous, subcutaneous, intramuscular, intratumoral, transnasal administrations, and by transmucosal application.

The complex according to the invention, nucleic acid molecules coding

20   therefore and/or cells or *in vitro* translation systems can be used for the preparation of a medicament for treating tumor diseases, allergies, autoimmune diseases, and chronic/acute inflammation reactions.

25   Results

Following the construction of three types of recombinant complexes (immunokinases), first results obtained demonstrate their superior quality with regard to binding specificity as well as cytoxicity.

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Construction and expression of a recombinant complex (immunokinase)

PCR-amplified DAPK2' DNA (Fig. 1) was directionally cloned into the ampicillin-resistant pMS-(L-ANG-Ki-4)-III/G eukaryotic expression vector containing a *Igk*-leader (L) sequence at the N-terminus, Ki-4(scFv) (component A) and a

5   tandem Myc- and His-Tag epitope at the C-terminus of the expression cassette (Fig. 2) Successful cloning was verified by DNA sequence analysis. Three days after transfection of 293T-cells, the appropriate sized expected recombinant complex (immuno-kinase) pMS-(L-DAPk2-Ki-4)-III/G (M_r ~66,000) was detected by Western blot analysis of protein mini-preparations. Transfected

10   producer-cells were further cultivated under Zeocin selection pressure in medium culture flasks and were used for larger scale production of the recombinant complex (immunokinase) pMS-(L-DAPk2-Ki-4)-III/G. Under normal culture conditions, between 0.1 and 0.5 μg of the recombinant protein were purified from 1 ml cell culture supernatant by a one step Ni-NTA

15   purification procedure. The intact recombinant complex (immunokinase) was secreted into the supernatant of transfected 293T-cells, as visualized by immunoblot using mouse-anti-penta-His monoclonal antibody.

PCR-amplified eEF-2K DNA encoding component B (Fig. 1, 4a-e) was

20   directionally cloned into the pET-derived kanamycin-resistant pBM-Ki-4(scFv) prokaryotic expression vector containing an IPTG-inducible *lac* operator, a *pelB* signal peptide followed by an enterokinase-cleavable His_{10} tag, and Ki-4(scFv) (component A) (Fig. 2). Successful cloning of the recombinant complex construct pMT-Ki-4(scFv)-eEF-2K was verified by DNA sequence analysis.

25   After transformation, recombinant *E.coli* BL21 Star™ (DE3) clones were cultivated under osmotic stress conditions in the presence of compatible solutes. The recombinant complex (immunokinase) was directed into the periplasmic space and the functional pMT-Ki-4(scFv)-eEF-2K (M_r ~113,000) protein directly purified by combination of IMAC and SEC to >90% purity. At

30   least 1 mg of purified pMT-Ki-4(scFv)-eEF-2K protein was routinely prepared from 1 liter of bacterial shaking cultures. The intact recombinant complex

(immunokinase) was secreted to the periplasmic compartment, as visualized by immunoblot using mouse-anti-penta-His monoclonal antibody.

Binding properties of recombinant complexes (immunokinases)

5   Fusing the Ki-4(scFv) coding regions, component A of the complex, to the kinase coding sequences, component B of the complex, did not affect the binding activity of the V_H/V_L antibody format of component A. Component A conferred specificity against the CD30 molecule. The purified recombinant complex (immunokinase) comprising the anti-CD30 component A always

10 bound to the Hodgkin-derived cell line L540Cy as measured by flow cytometry (Fig. 3).

In vitro cytotoxic activity

To characterize the cytotoxic activity of the recombinant complex comprising

15 anti-CD30 (as component A) and kinases (component B) *in vitro*, the proliferation of different target cells was evaluated after incubation with different amounts of the recombinant complexes (immunokinases) pMS-(L-DAPk2-Ki-4)-III/G and pMT-Ki-4(scFv)-eEF-2K, respectively. Growth inhibition of the CD30-positive cell lines L540Cy and HL60 were documented by a XTT-

20 based colorimetric assay. Toxic effects were observed only against CD30-positive cells with a calculated median IC_{50} of betwenn 4 and 35 ng/ml on L540Cy cells (Fig. 4) The CD30-negative Ramos and 8701-BC cell lines were not affected by recombinant immunokinase concentrations of up to 10 µg/ml. Thus the component A (anti-CD30 scFv) of the complex conferred specificity to

25 the recombinant complex, limiting the cytotoxic effects of the kinase domain to the selected target cells.

Examples

Bacterial strains, oligonucleotides, and plasmids

30 *E.coli* XL1-blue (supE44 hsdR17 recA1 endA1 gyr A46 thi relA1 lacF'[pro AB$^+$ lacI^q lacZ ?M15 Tn10(tet^r)]) were used for the propagation of plasmids, and

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E.coli BL21 Star™ (DE3) (F⁻ ompT hsdS$_B$(rB⁻m B⁻) gal dcm rne131 DE3) as host for synthesis of recombinant immunokinases. Synthetic oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). The bacterial expression vector pBM-Ki-4 is derived from the pET27b plasmid (Novagen,

5   Madison, USA), and is used for the expression of the C-terminal fusion of Not I/Blp I-kinase domains to the anti-CD30 scFv (Klimka, A. *et al.*, 1999). The eukaryotic expression vectors pMSKAngII and pMSLAngKIII are derived from the pSecTag plasmid (Invitrogen, Carlsbad, USA) and are used for N- or C- terminal fusion of XbaI/BlpI-kinase domains to the Ki-4(scFv) (Stöcker, M. *et*

10 *al.*, 2003). Plasmids were prepared by the alkaline lysis method and purified using plasmid preparation kits from Qiagen (Hilden, Germany). Restriction fragments or PCR products were separated by horizontal agarose gel electrophoresis and extracted with QIAquick (Qiagen). All standard cloning procedures were carried out as described by Sambrook, J. et al., 1989.

15

Cell culture

All cell lines, including the CD30-positive cell lines L540Cy (Kapp, U. et al., 1992) and HL-60 (Thepen, T. Utrecht, The Netherlands) the CD30-negative cell lines Ramos (ATCC, VA, USA) and 8701-BC (Minafra, S. et al., 1989) and

20 the producer cell line 293T (ATCC) were cultivated in complex medium (RPMI 1640) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. All cells were cultured at 37°C in a 5% CO_2 in air atmosphere. For the selection of transfected cells, Zeocin (Invitrogen) was added to a final concentration of 100

25 µg/ml.

Construction and expression of recombinant complexes (immunokinases)

Cloning and expression of pMS-(L-DAPk2-Ki-4)-III/G (SEQ ID NO 1) and pMS-

30 *(Ki-4-DAPk2)-II/G (SEQ ID NO 3)*

For the construction of a vector encoding a recombinant complex with N- or C-terminal DAP-kinase 2 (DAPk2)-fusions, DAPk2 was PCR amplified to

introduce the restriction sites *XbaI* and *BlpI*. After *XbaI/BlpI*-digestion, the PCR-product was cloned into the eukaryotic expression vector pMS-(L-ANG-Ki-4)-III/G and pMS-(Ki-4-ANG)-II/G respectively, digested with the same restriction enzymes. The resulting recombinant constructs pMS-(L-DAPk2-Ki-4)-III/G (SEQ ID NO: 1) and pMS-(Ki-4-DAPk2)-II/G (SEQ ID NO: 3) encoding the immukinase proteins L-DAPk2-Ki-4-MH (SEQ ID NO 2) and L-Ki-4-DAPk2-MH (SEQ ID NO 4) were verified by sequence analysis. After *TransFast*-mediated (Promega, Mannhein, Germany) transformation into 293T-cells, the recombinant immunokinase was expressed as described by Stöcker M. *et al.*, 2003. Briefly, one μg plasmid-DNA and 3μl TransFast have been used according to the manufactures protocol for 12 well cell culture plates. Transfection efficiency was between 75 and 95% determined by counting green fluorescent cells. 3 days after initial transfection, cell culture supernatants were analyzed for recombinant protein. Subsequently, transfected cells were transferred into medium-sized cell culture flasks (Nunc; 85m²) and grown in RPMI complex medium supplemented with 100 μg/ml Zeocin. One to two weeks productively transfected clones were green fluorescing and hence could be detected by fluorescence microscopy. Transfected cell populations were established by subcultivation of these clones. Purifications of the His-tagged proteins were accomplished by the Ni-NTA metal-affinity method (Hochuli, V., 1989, Porath, J. *et al.*, 1975) (Qiagen). The protein purification followed a modified protocol for the purification of native protein from Qiagen (*The Expressionist* 07/97). For protein mini-preparation, 900 μl centrifugation-cleared cell culture supernatant was supplemented with 300μl of 4x incubation buffer (200mM NaH_2PO_4, pH 8.0; 1.2M NaCl; 40mM Imidazol) and 30μl 50% Ni-NTA. Following 1h incubation, the Ni-NTA resin was pelleted by centrifugation. After washing the sediment twice in 175 μl 1x incubation buffer, bound protein was eluted with 30 μl of elution buffer (50mM NaH_2PO_4, pH 8.0; 1.2M NaCl; and 40 mM imidazol) and 30μl 50% Ni-NTA. Following an 1 h incubation, the Ni-NTA resin was pelleted by centrifugation. After washing the sediment twice in 175 μl 1x incubation buffer, bound protein was eluted with 30 μl of elution buffer (50mM

NaH$_2$PO$_4$, pH8.0; 300mM NaCl; 250mM Imidazol) for 20min at RT. Larger scale purification of eukaryotically-expressed proteins up to 500ml cell culture supernatant was performed on a BioLogic workstation (Bio-Rad, USA). Cell culture supernatants were loaded onto a Ni-NTA column and following elution
5   of the His-tagged proteins were made under the conditions described above.

Cloning and expression of pMT-Ki-4(scFv)-eEF-2K
The eukaryotic elongation factor-2 kinase (eEF-2k) was amplified by PCR to introduce the restriction sites *NotI* and *BlpI*. After *NotI/BlpI*-digestion, the
10   PCR-fragment was cloned into the bacterial expression vector pBM-Ki-4, digested with the same restriction enzymes. The resulting recombinant construct pMT-Ki-4(scFv)-eEF-2K (SEQ ID NO: 5) was verified by DNA sequence analysis. After transformation into BL21 Star™ (DE3), the immunokinase Ki-4(scFv)-eEF-2K (SEQ ID NO 6) were periplasmically
15   expressed under osmotic stress in the presence of compatible solutes as described by Barth, S. *et al.* 2000. Briefly, transformed bacteria were harvested 15 h after IPTG induction. The bacterial pellet was resuspended in sonication-buffer (75 mM Tris/HCl (pH 8), 300 mM NaCl, 1 capsule of protease inhibitors/ 50 ml (Complete™, Roche Diagnostics, Mannheim, Germany), 5 mM
20   DTT, 10 mM EDTA, 10% (v/v) glycerol) at 4°C and sonicated 6 times for 30 s at 200 W. The 'm22(scFv)-ETA' fusion proteins were enriched by IMAC (immobilized metal-ion affinity chromatography) using nickel-nitriloacetic chelating Sepharose (Qiagen) and SEC (size exclusion chromatography) with Bio-Prep SE-100/17 (Biorad, München, Germany) columns according to the
25   manufacturer's instructions. Recombinant Protein was eluted with PBS (pH 7.4) and 1 M NaCl, analyzed by Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), quantified by densitometry (GS-700 Imaging Densitometer; Biorad) after Coomassie staining in comparison with BSA standards and verified by Bradford assays (Biorad).
30

SDS-PAGE and Western Blot Analysis

SDS-PAGE, Coomassie staining, and Western blotting were performed as described by Barth, S. et al., 1998. Briefly, recombinant His-tagged immunokinases were detected by mouse-anti-penta-His moab (Qiagen). Bound antibody was detected by a horseradish-conjugated donkey-anti-mouse-IgG moab (Dianova, Hamburg, Germany), followed by ECL-mediated (Amersham Biosciences, Freiburg, Germany), chemiluminescence reaction and exposition to appropriate X-ray film (Roche, Penzberg, Germany) or alkaline-phosphatase-conjugated anti-mouse-IgG moab (Sigma Chemical Co., Deisenhofen, Germany) and a solution of Tris-HCl (pH 8.0) and 0.2 mg/ml naphtol-AS-Bi-phosphate (Sigma Chemical Co.) supplemented with 1 mg/ml Fast-Red (Serva, Heidelberg, Germany).

Cell membrane (CM) ELISA

The binding activity of recombinant complexes (immunokinases) were determined by CM-ELISA using biological active membranes of tumor cells as described recently by Tur, MK. et al., 2003. Briefly, ELISA Maxisorp-Plates (Nalge Nunc International, Roskilde, Denmark) were coated with 100 µl (~ 0.9 mg protein/ml) freshly prepared membrane fractions of CD30-positive L540Cy/HL60 cells and Ramos/8701-BC as control in 0.02 M bicarbonate buffer, pH 9.6, overnight at 4°C. Plates were washed five times with PBS (pH 7.4) containing 0.2% Tween 20 (TPBS) and blocked with 200 µl 2% BSA in PBS. After overnight incubation at 4°C, plates were washed five times with TPBS and 1 - 10 µg/ml of recombinant immunokinases diluted with 0.5% BSA (w/v) and 0.05% Tween 20 (v/v) in PBS was added to the plates and incubated at RT (23°C) for 1h. Peroxidase labeled anti-His IgG conjugate (Qiagen) were added diluted with 0.5% BSA and 0.05% Tween 20 in PBS according to manufactures instructions. Bound antibodies were visualized after addition of 100 µl 2', 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution (Roche Molecular Biochemical's, Mannheim, Germany) by measuring the extinction at 415 nm with an ELISA-Reader (MWG Biotech).

Flow cytometry analyses

Cell binding activity of the recombinant complexes (immunokinases) expressed in *E.coli* BL21 Star™ (DE3) was evaluated using a FACSCalibur flow cytometry instrument and CellQuest software (Becton Dickinson, Heidelberg,

5   Germany). Cells were stained with recombinant protein as described (25). Briefly, ten thousand events were collected for each sample, and analyses of intact cells were performed using appropriate scatter gates to exclude cellular debris and aggregates. 5×10^5 cells were incubated for 1 h on ice with 50 µl of bacterial protein extract at a concentration of 30-40 µg/ml or 100 µl of the

10   immunokuinase containing supernatants respectively. The cells were washed with PBS buffer containing 0.2% w/v BSA and 0.05% w/v sodium azide (PBA) and then incubated for 30 min with anti-penta-His moab (Qiagen) diluted 1:2 in PBA buffer. Cells were washed and incubated with fluorescein-iso-thiocyanate (FITC)-labeled goat-anti-mouse IgG (DAKO Diagnostica,

15   Hamburg, Germany) for 1h at 4°C. After a final wash, the cells were treated with 2µl 6.25 mg/ml propidiumiodide and subsequently analyzed on a FACScalibur (Becton Dickison, Heidelberg, Germany).

Colorimetric cell proliferation assay

20   The cytotoxic effect of the recombinant complexes (immunokinases) on target cells was determined by measurement of metabolization of yellow tetrazolium salt (XTT) to a water soluble orange formazan dye was determined as published by Barth, S. et al. 2000. Various dilutions of the recombinant immunokinase were distributed in 100 µl-aliquots in 96-well plates. Two-four x

25   10^4 target cells in 100 µl aliquots of complete medium were added and the plates were incubated for 48 h at 37°C. Afterwards, the cell cultures were pulsed with 100 µl fresh culture medium supplemented with XTT/PMS (final concentrations of 0.3 mg and 0.383 ng respectively) for 4 h. The spectrophotometrical absorbances of the samples were measured at 450 and

30   650 nm (reference wavelength) with an ELISA reader (MWG Biotech). The concentration required to achieve a 50% reduction of protein synthesis (IC_{50}) relative to untreated control cells and to 1% Triton X treated positive controls

was calculated graphically via Excel generated diagrams. All measurements were done in triplicate.

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